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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The androgen receptor (AR) binds direct repeats as well as inverted repeats of the 5'-AGAACA-3' core element. This makes this receptor unique in the family of nuclear receptors. The inverted repeats are called canonical AREs, the direct repeats selective AREs. The high affinity binding to the selective AREs involves a carboxyterminal extension (CTE) of the DNA-binding domain (DBD). We are expressing large amounts of AR fragments to clarify the structure of the AR-DBD + CTE bound to these AREs by X-ray diffractions on co-crystals. The latter will be done in collaboration with Dr. Daniel Gewirth (cfr DAMD17-01-1-0050).

In addition, the CTE has been reported to be involved in the transactivating properties of the AR. We have determined minimal deletions in the hinge region which result in a stronger activating AR. Surprisingly, these deletions have a strong negative effect on DNA-binding. Moreover, other deletions result in an AR which is less active (compared to wild type AR) on some AREs. A series of point mutations is being finalized and tested for discriminating mutations which affect only the DNA-binding, the potentiation or the repression of transactivation of the AR.

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The hinge region as a key regulatory element of androgen receptor dimerization, DNA binding and transactivation

Introduction

Androgens play key roles, not only in male development, but also in many normal physiological processes in both sexes and in the development and progression of prostate cancer. The androgen receptor is a member of the family of transcription factors called nuclear receptors from which a subfamily, called class I receptors, recognize very similar DNA sequences. This class I consists of the steroid receptors for glucocorticoids, progestagens, mineralocorticoids and androgens, which all can bind bipartite hormone response elements organized as inverted repeats of 5'-AGAACA-3'-like core elements separated by three nucleotides. More recently, however, we discovered that the androgen receptor can bind direct repeats of the same core elements as well. This makes this receptor unique in the family of nuclear receptors, although other receptors, like the vitamine D receptor also recognize direct repeats, but in this case it is a repeat of a different core sequence 5'-AGGTCA-3'. The inverted repeats are called canonical AREs, while the direct repeats will be called selective AREs. Two zinc coordinating modules that constitute the receptors DNA-binding domain, are involved in the recognition of canonical AREs, but in the high affinity binding to the selective AREs, a carboxyterminal extension (CTE) is needed. The subject of the here reported research, is to determine the exact structure of the DNA-binding domain bound to direct repeats versus inverted repeats. We are over-expressing androgen receptor fragments in an attempt to clarify the structure, not only of the hinge region, but also of larger receptor fragments. The latter will be done in collaboration with Dr. Daniel Gewirth (cfr DAMD17-01-1-0050).

This CTE is not only involved in DNA binding, but is also the acceptor for posttranslational modifications like phosphorylation and acetylation. In addition, the CTE has been reported to be involved in nuclear translocation as well as the transactivating properties of the androgen receptor. We are unraveling the structure-function relationships in the CTE by deletion and point mutation analyses. Mutated receptors and receptor fragments are being tested in DNA-binding, transactivation assays in mammalian cells on selective and canonical ARE-containing reporter constructs as well as in yeast.

We have attracted a PhD student from South Africa, Tamzin Tanner, to perform the experimental work. Due to slower administration, her visum/work permit was only completed by the end of September 2002. However, in collaboration with other members of the laboratory, she managed to complete a large part of the tasks put forward in the first year of the 'statement of work'.

Body

A. Analysis of crystals

This part of the work is ongoing in the laboratory of Dr. Daniel Gewirth (see Annual report Grant DAMD17-01-1-0050). The optimalisation of the crystallization is entirely done in his laboratory with the help of mutated DNA-binding domains and oligonucleotides containing androgen response elements.

We are currently constructing larger fragments of the androgen receptor (all containing the DNA-binding domain, figure 1) as fusion to glutathion S-transferase (GST) which should enable us to produce larger amounts in *E.coli.* The constructs have been partially sequenced at the moment of writing.

Figure 2 shows an example of a GST-fusion of the NTD-DBD fragment of the human androgen receptor, in which the glutamine repeat has been deleted, expressed in *E.coli.* Although the expressed protein is visible after Coomassie staining, we will optimize the expression and purification of this fragment further. Similarly, we are cloning DBD-LBD fragments (with and without the hinge region) as GST fusion proteins for overexpression in E.coli. When appropriate expression conditions are found, we will verify DNA-binding and when appropriate, hormone binding. We will introduce the Cys552 to Alanine substitution for which Gewirth and collaborators have shown a better crystallization.

In a parallel program, we have analyzed several features of the amino-terminal domain: its interaction with co-activators, with the ligand-binding domain, and its transactivating properties (Christiaens *et al.* 2002; Callewaert *et al.* 2003). In addition, the amino-terminal domain of the androgen receptor contains two sumoylation sites. These are Lysines to which a small ubiquitin-like modifier can be attached, which have earlier been described as synergy control elements. This post-translational modification involves Ubc9 as a SUMO-ligase, which has been described as a hinge region-binding protein by the group of Olli Jänne. In double hybrid analyses (results not shown), Ubc9 binds the receptor. Surprisingly, when the hinge region is deleted, Ubc9 still interacts with the receptor, demonstrating that alternative binding sites are present elsewhere. We are currently determining the other Ubc9-binding sites in the receptor, as well as study the effects of mutating the sumoylation acceptor sites on the receptors activities.

B. Deletion of the hinge region

In figure 3, a schematic representation of the first generation deletion constructs that have been developed at the moment of writing is shown. The indicated receptor derivatives have been cloned in the mammalian expression vector pSG5 in frame with a Flag-tag coding sequence. The latter will enable an efficient immunologic detection with commercial anti-Flag antibody (M2). Only the upper clones (named Δ1 to Δ5) have been studied thus far. The sequence of the other constructs is being analyzed.

-Role of the hinge region in sequence-specific binding

The hinge region plays a critical role in high affinity DNA binding as measured by gel retardation assays. This function was reported for the isolated DNA-binding domain (reviewed in

Claessens and Gewirth, *submitted*). The effects of deletions in the CTE on DNA-binding of the isolated DNA-binding domain expressed in *E.coli* (Schoenmakers *et al.* 1999) are similar to the effects on the full size receptor expressed in mammalian cells (Figure 4). Indeed, deletion of the complete CTE (cfr Δ1) abolished DNA-binding, readdition of four residues (cfr. Δ2) restored binding to the C3(1)ARE and re-addition of nine residues Δ3 restored affinity for both the C3(1)ARE and the PB-ARE-2. Remark that the DNA-binding by the receptors with a deleted CTE (Δ1 and Δ2) is barely visible, even after longer exposures of the gel.

- Inhibition of transactivation

Wang *et al.* 2000 reported that a deletion of part of the hinge region relieved repression of the activation function 2 of the androgen receptor. Our experimental data (not shown) in yeast as well as in mammalian cells do **not** corroborate this report since the AR DBD-LBD fragment does **not** transactivate our reporter constructs in transient transfection experiments, irrespective whether the hinge region is present or (partially) deleted.

When, however, deletions were made in the context of the full size receptor, they did dramatically affect the receptors activity. The deletion of residues 628 to 646 resulted in a three to five-fold increase of the androgen responses mediated via the C3(1)ARE, a canonical ARE, or via a selective ARE (scARE) (figure 5).

The effect is observed in COS cells as well as in Hela cells, other cell lines (e.g. LNCaP cells) will be tested to detect cell line specificities of the here reported effects.

Surprisingly, the effects we observed on DNA binding and transactivation were not correlated. When the deletion affected DNA-binding severely (up to a point where DNA-binding is barely detectable), the activation of reporter genes was enhanced. Clearly the deleted fragments must be involved in at least two different functions: sequence-specific DNA-binding on the one hand, and an inhibition of transactivation on the other. The exact mechanism(s) of this inhibition of transactivation is now under investigation.

Structure function relation of transactivation control by the hinge region

From the other deletion constructs, we learned that adding back 4 amino acids of the CTE abolished the effect of CTE-removal in the transactivation, as well as the DNA-binding assays. In the case of the MMTV reporter (figure 6), adding back more than 4 residues resulted in a less active receptor. This effect, which is not so prominent on the other reporters (figure 5), points out that here to two different mechanisms are working: one positive, acting via the first four residues and one negative acting via the next part of the CTE.

A scanning mutation analysis of the hinge region is under way. In figure 7, the residues which have been changed into valine are indicated, as well as the preliminary determination of the relative activities of these constructs. Clearly, single substitutions are not as effective as the deletions and further study is being performed.

C. Start the study of AR Gal4-LBD in yeast

We have constructed an expression vector containing a fusion of the Gal4 DNA-binding domain with the AR LBD fragment from position 628 to 917. In this construct we deleted parts of the hinge region from position 628 to 646 or 632 to 646. In the yeast strain Y190, we could not detect transactivating properties of any of these constructs. This is in clear contrast with earlier reports (Wang *et al.* 2000). As a positive control the full size AR was fused to the Gal4 DBD. This construct is clearly ligand-responsive. Since we observed earlier that a fusion of the AR-LBD with a heterologous DBD acted differently from a fusion with its own DBD, we constructed a fusion containing the Gal4 DBD fused to the AR DBD-LBD fragment. This fusion was also inactive in our yeast assays, even when the hinge region (fragment 628-646) was deleted.

Nonetheless, these contructs will become useful for double hybrid analyses in yeast of the interactions with p160s and other coactivators.

In our working hypothesis, however, we propose allosteric effects of the AREs, not only on the DNA-binding domains, but also on other domains/functions of the AR. We plan to replace the UAS reporter constructs in the yeast genome by ARE reporter constructs. This would enable the analysis of the activity of the AR fragments when the own DBD is used.

Figure legends

Figure 1

Schematic representation of the androgen receptor with indicated amino-terminal domain (NTD), central DNA-binding domain (DBD) hinge region (H) and ligand-binding domain (LBD). A glutamine repeat in the amino-terminal domain is also indicated.

The fragments for which the reading frames have been cloned in pGEX vector are indicated.

Figure 2

Results of the first expression experiment of two clones expressing the fusion of the amino-terminal domain (with deleted Q-repeat), indicated with an asterisk in figure 1. A saturated culture of *E.coli* (XL blue strain) containing the expression plasmid was diluted 10-fold and grown for 2 hrs. A sample of the culture was taken before and after addition of IPTG, and the samples were lysed in SDS-PAGE loading dye and separated by PAGE. After Coomassie staining, the IPTG induced band with the correct molecular weight has been indicated with arrows.

Figure 3

Schematic representation of the deletion constructs that have been made. The sequence of the hinge region is given and the position of the deleted residues is indicated by the boxes. The constructs analyzed at this moment are WT: wild type receptor; Δ1 corresponds with the deletion of residues 628 to 646; Δ2 with deletion of 629 to 646;Δ3 with deletion of 632 to 646; Δ4 with deletion of 637 to 646 and Δ5 with deletion of 640-646.

All constructs have been cloned in the mammalian expression vector pSG5 with the addition of a Flag-tag for convenient immuno-detection.

Figure 4

Gel retardation experiments. The constructs named in figure 3 were transfected in COS7 cells and equal amounts of extracts were incubated with radiolabelled canonical ARE C3(1)ARE (left panel) or the selective PB-ARE-2 (right panel), before the complexed © and free DNA probes (FP) were separated in a non-denaturing electrophoresis. The specificity of the binding, was demonstrated by the supershifts (SS) induced by addition of antibodies raised against the androgen receptor.

Figure 5

Functional analyses of the constructs named in figure 3. The expression plasmids were co-transfected together with a betagalactosidase expression vector (control), a luciferase reporter construct under control of the indicated ARE. The cells were incubated with vehicle or 10^{-8} M R1881, an androgen receptor agonist for 24 hrs before extracts were examined for luciferase and betagalactosidase activity. The data were calculated induction folds of luciferase activities by the hormone. S.E.M. from three independent experiments are indicated.

Top panels show results of activation of a reporter containing the C3(1)ARE in COS7 (right) and HeLa cells (left). The lower panel shows results for a reporter containing the scare.

Figure 6

Activation of the hormone responsive enhancer of the mouse mammary tumour long terminal repeat by androgens mediated through the indicated constructs (described in figure 1). Results are obtained cfr figure 5.

Figure 7

Point mutations have been introduced resulting in single amino-acid substitutions in the hinge region of the AR. The residues changed into Valine are indicated. The preliminary results of functional assays, performed as described in figure 6 are depicted in the lower panel.

Please remark that these experiments will be repeated in order to decrease the S.E.M.

Figure 1: GST-fusion constructs of AR fragments

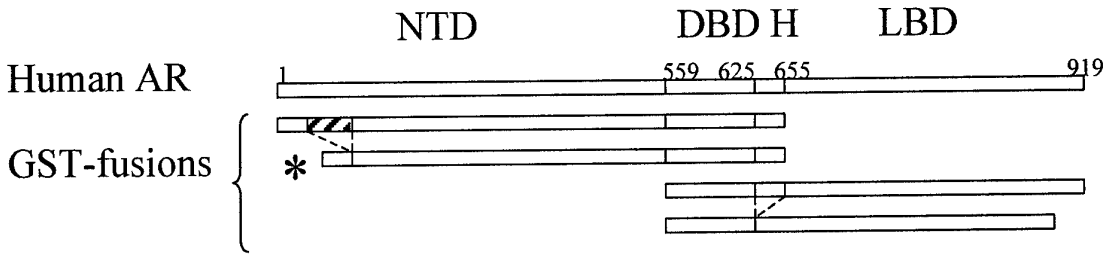


Figure 2: Expression of GST-fusions in *E. coli*

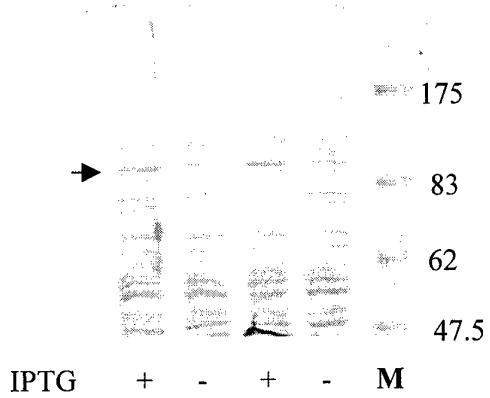


Figure 3 : Deletions in the hinge region

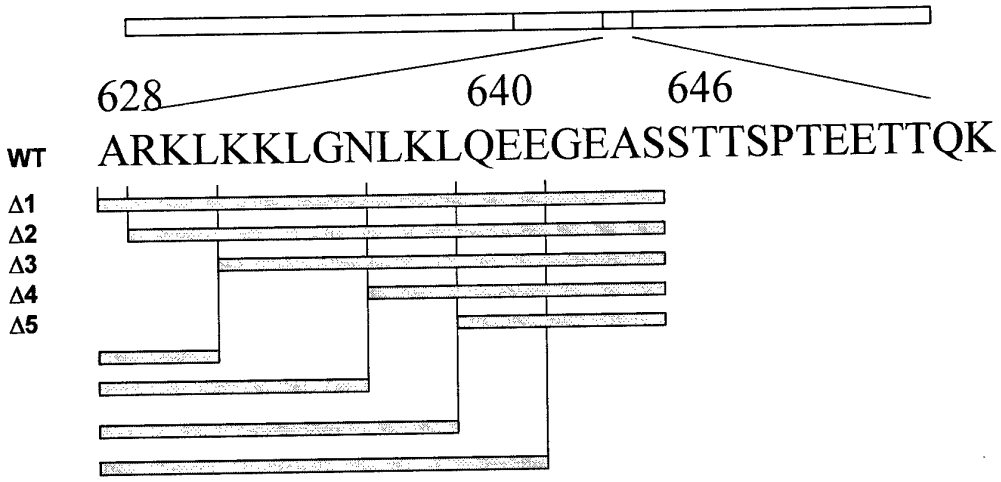


Figure 4: DNA binding assays

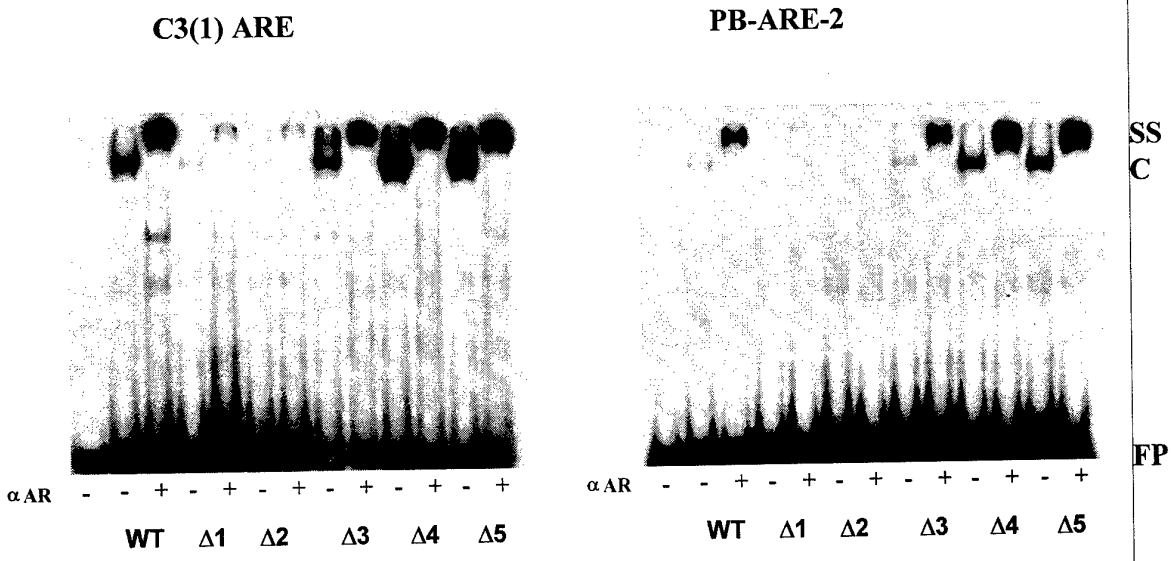


Figure 5: Functional analyses of CTE deletions on a canonical and a selective ARE

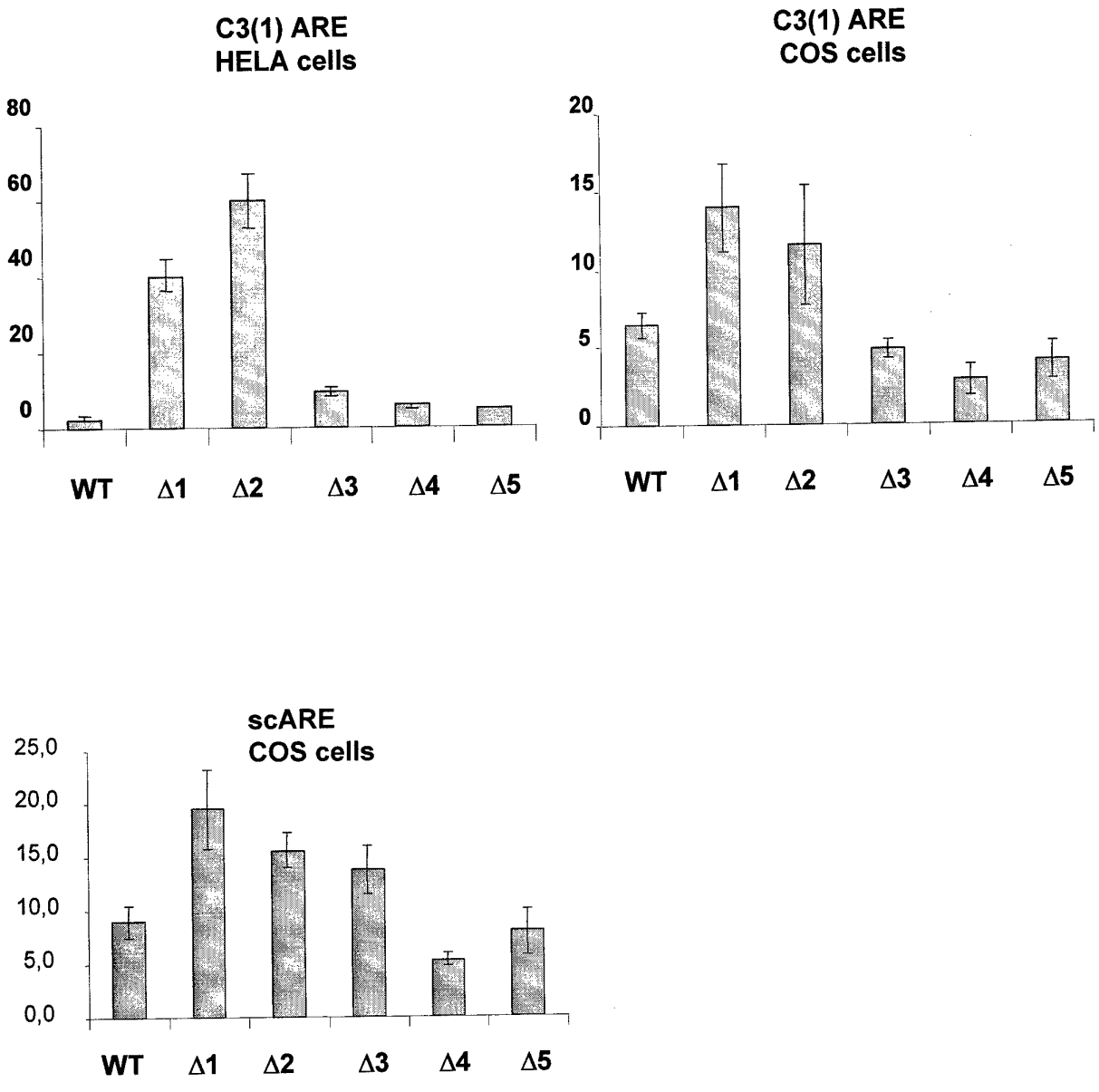


Figure 6: Activation of a MMTV-based repoter gene by deltion constructs of the androgen receptor

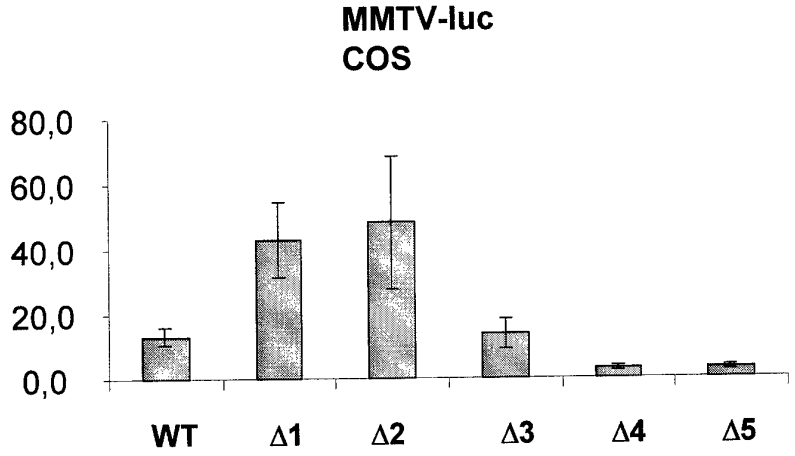
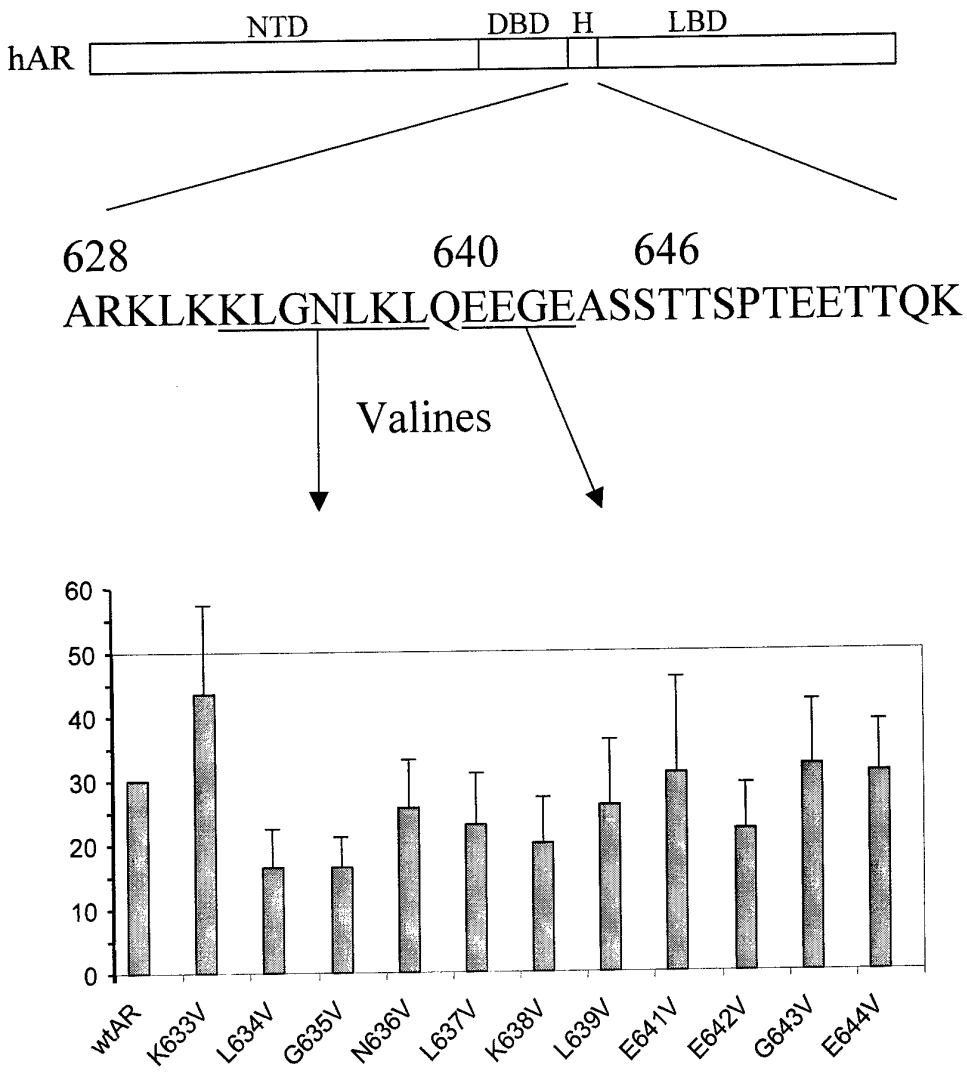


Figure 7 : Functional analyses of point mutations in the hinge region



Key Research accomplishments

- We have constructed expression vectors for fusion proteins of glutathion S-transferase with AR fragments for overexpression in *E. coli*. Deletions of the hinge region and of the glutamine-repeat in the amino-terminal domain have been constructed.

- We made a series of deletions and point mutations in the hinge region in full size AR, as well as the DBD-LBD fragment.

- We established the effect of deletions on DNA-binding by the full size androgen receptor.

- We established the effects on transactivation by the androgen receptor via different androgen responsive enhancers.

- We established mutations which discriminate between 'repression of transactivation' and 'super activation'.

- A series of single point mutations have been constructed and are being analysed. None of them seem to mimic the effects of repression or activation.

Reportable outcomes

Further analyses are certainly required before the structure function analyses reported here can be submitted for publications.

We plan publications in international journals during the following year. An oral presentation at the next FEBS Meeting in Brussels (june 2003) will partially cover data from this report.

Conclusions

- GST-fusions of several AR-fragments are well expressed in *E. coli.* This is a prerequisite for further structural analyses. The expression and purifications will be optimized in the next year.

- Although the effect of deletion of the hinge region (Wang *et al.* 2000) in the DBD-LBD construct could not be repeated, the deletion in the full size AR (containing the amino-terminal domain) was very clear.

-Much to our surprise, the receptor constructs Δ1 and Δ2 display a very low affinity for DNA but are even more potent in the transactivation assays. It seems that this can not simply be explained by a strong enhancement of the AF2, since in our hands the AR DBD-LBD construct is inactive irrespective of whether the hinge region is deleted or not. Alternative explanations, AF1 activity, recruitment of other co-activators or loss of binding of corepressors, receptor concentration, degradation via the proteasome or being studied.

-Analyses of smaller deletions constructs confirmed the complexity of the structure-function relationships within the CTE and the hinge region of the AR. We have made several observations which need further investigation and have set up the necessary assays. The point mutation analyses will be extended to mutations to other types of residues and combination of two or more mutations.

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